SeqCap EZ HyperCap Workflow User's Guide

Version 2.3

For Research Use Only.
Not for use in diagnostic procedures.

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Editions

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Preface

Regulatory Disclaimer

For Research Use Only. Not for use in diagnostic procedures.

SeqCap EZ Probe Pool

SeqCap EZ probe pool is a solution-based capture reagent that enables enrichment of the whole exome or customer regions of interest in a single tube. Throughout this document, 'SeqCap EZ probe pool' refers to the SeqCap EZ, SeqCap EZ Prime, and SeqCap EZ probes.

Contact Information

Technical Support

If you have questions, contact your local Roche Technical Support. Go to *sequencing.roche.com/support.html* for contact information.

Manufacturer and Distribution

Manufacturer	Roche Sequencing Solutions, Inc. Pleasanton, CA USA
Distribution	Roche Diagnostics GmbH Mannheim, Germany
Distribution in USA	Roche Diagnostics Corporation Indianapolis, IN USA

Conventions Used in This Manual

Symbols

Symbol	Description
	Important Note: Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.

Text

Conventions	Description
Numbered listing	Indicates steps in a procedure that must be performed in the order listed.
Italic type, blue	Identifies a resource in a different area of this manual or on a web site.
Italic type	Identifies the names of dialog boxes, windows, tabs, panels, views, or message boxes in the software.
Bold type	Identifies names of menus and controls (buttons, checkboxes, *etc.*) in the software.

Chapter 1. Before You Begin

This *User's Guide* describes the process for enrichment of individual or multiplexed genomic DNA (gDNA) sample libraries using any SeqCap EZ probes, and the amplification of these sample libraries by ligation-mediated PCR (LM-PCR). Specifically, this *User's Guide* provides a protocol for the workflow outlined in *Figure 1* using the KAPA Hyper Prep Kit or KAPA HyperPlus Library Preparation Kit. The output of this protocol consists of enriched gDNA fragments that can be sequenced directly using an Illumina sequencing instrument.

The *SeqCap EZ HyperCap Workflow User's Guide* includes detailed steps for how to generate a DNA sample library. For optimal performance with Roche targeted enrichment, the library preparation steps as detailed in this *User's Guide* should be used for the library preparation as they have been optimized for this workflow.

Benefits of this *User's Guide* include:

- The KAPA Hyper Prep and KAPA HyperPlus Library Preparation Kit workflows provide a novel one-tube chemistry and protocol that increase library complexity while reducing workflow time and hands on time. For additional information on library preparation, refer to the Important Parameters section in the most current version of the KAPA Hyper Prep Kit Technical Data Sheet, KR0961, and the KAPA HyperPlus Kit Technical Data Sheet, KR1145.
    - The KAPA Hyper Prep Kit allows for mechanical shearing of gDNA using a Covaris Focused-ultrasonicator.
    - The KAPA HyperPlus Library Preparation Kit enables enzymatic fragmentation of gDNA.
- Reduction in required equipment. (Vacuum centrifuge and water bath are no longer required. A Covaris ultrasonicator is no longer required if using the KAPA HyperPlus Library Preparation Kit).
- Additional workflow improvements include reduced workflow time downstream of library preparation, and increased compatibility of steps with automated liquid handlers.

The SeqCap EZ HyperCap workflow is designed to provide flexibility for adapting sequence capture to meet a broad range of sample, workflow and experimental needs. While not recommended for general use, modification of certain protocol steps may be appropriate for individual circumstances. Contact Technical Support at *sequencing.roche.com/support.html* for more information on the following:

- Input of very low amounts of DNA or poor quality DNA, into library preparation (see also troubleshooting section in *Appendix D*).
- Customization of PCR cycle number and amount of amplified sample library put into hybridization for improving sequencing metrics (see also troubleshooting section in *Appendix D*).
- Omission of the double sided size selection step during library preparation, especially in instances where sample complexity is important such as low DNA input or low DNA quality input into library preparation (see also troubleshooting section in *Appendix D* for recommendations and considerations).
- Latest automation protocols for various liquid handlers and developments on automation friendly options.
- Reduction in hybridization time to below the recommended 16 to 20 hours.

Workflow

The SeqCap EZ HyperCap workflow (*Figure 1*) involves:

1. Preparation of the gDNA sample library using a KAPA Hyper Prep Kit or KAPA HyperPlus Library Preparation Kit.
2. Amplification of the DNA sample library using KAPA HiFi HotStart ReadyMix (2X).
3. Capturing target regions by hybridizing the gDNA sample library with the SeqCap EZ probe pool.
4. Recovery of captured sample using the HyperCap Target Enrichment Kit.
5. Amplification of the captured DNA sample using KAPA HiFi HotStart ReadyMix (2X).
6. Sequencing the captured and amplified DNA sample using an Illumina sequencing instrument.

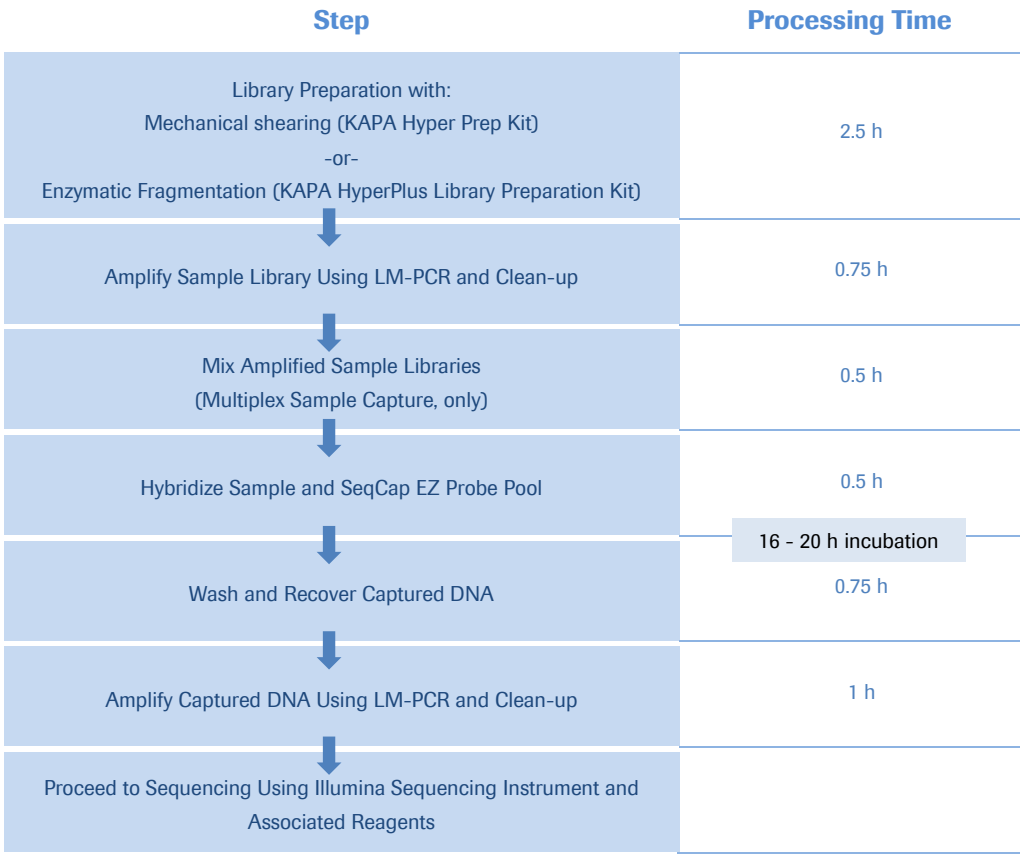


Figure 1: SeqCap EZ HyperCap workflows, with mechanical or enzymatic DNA fragmentation. Where applicable, incubation times are indicated between steps.

Prepare the Following Reagents and Equipment

- Thermocyclers should be programmed with the required thermocycler programs:
  - Pre-Capture LM-PCR program (*Chapter 4*, Step 2.2)
  - Hybridization incubation program (*Chapter 5*, Step 3.22)
  - Post-Capture LM-PCR program (*Chapter 7*, Step 3.2)

It is recommended to use a thermocycler with a heated lid, which tracks +10 °C above the incubation temperature.

- The following reagents should be prepared as described before beginning the protocol:
  - Aliquot SeqCap EZ Probe Pool (*Chapter 2*, Step 1)
  - Resuspend Index Adapters (*Chapter 3*, Step 1)
  - Resuspend Post-LM-PCR Oligos (*Chapter 7*, Step 1)

What's New in v2.3?

- Updated the microliter font to resolve PDF rendering issues.
- Updated kit names and references to Universal Blocking Oligos.
- Corrected DynaMag-2 Magnet 16 x 0.2 mL tube holder to 16 x 2.0 mL tube holder.
- Updated kit names or references to components for SeqCap EZ.
- Added HyperCap as a Roche registered trademark.

To verify you are using the most up-to-date version of this *User's Guide* to process your captures, go to *sequencing.roche.com/support.html*.

Terminology

LM-PCR: Ligation Mediated PCR. In the context of this document, PCR using primers complementary to the sequencing adapters.

Sequence Capture (or Capture): The process of enriching targeted regions from genomic DNA. In the context of this document, the hybridization of the amplified sample library and SeqCap EZ probe pool, and subsequent washing steps.

SeqCap EZ probe pool: The complete set of biotinylated long oligonucleotide probes (SeqCap EZ Human Exome, SeqCap EZ MedExome, SeqCap EZ Choice, SeqCap EZ Choice XL, SeqCap EZ Developer, SeqCap EZ Prime Choice, SeqCap EZ Prime Choice XL, or SeqCap EZ Prime Developer) provided by Roche to perform sequence capture.

Sample Library: The initial shotgun library generated from genomic DNA by fragmentation and ligation of sequencing platform-specific linkers. In the context of this document, the sample library before amplification by LM-PCR and before capture.

Amplified Sample Library: The sample library after amplification by LM-PCR but before capture.

Captured Multiplex DNA Sample: The enriched DNA population from the amplified sample library after the multiplex capture process but before another round of LM-PCR.

UBO: Universal Blocking Oligos.

Amplified, Captured Multiplex DNA Sample: The captured DNA after LM-PCR amplification.

Components Supplied

Component	Description
SeqCap EZ MedExome Probes	Available in 4, 48, or 384 reaction packs
SeqCap EZ MedExome Plus Probes	Available in 48 reaction packs
SeqCap EZ Choice Probes SeqCap EZ Choice XL Probes SeqCap EZ Developer Probes SeqCap EZ Prime Choice Probes SeqCap EZ Prime Choice XL Probes SeqCap EZ Prime Developer Probes	Available in 4, 12, 24, 48, 96, 384 or 960 reaction packs
SeqCap EZ Share Choice Probes SeqCap EZ Share Choice XL Probes SeqCap EZ Share Developer Probes SeqCap EZ Share Prime Choice Probes SeqCap EZ Share Prime Choice XL Probes SeqCap EZ Share Prime Developer Probes	Available in 24, 96, 384 reaction packs
SeqCap EZ Human Exome Probes v3.0	Available in 4 or 48 reaction packs
SeqCap EZ Exome Plus Probes	Available in 12, 48 or 96 reaction packs
SeqCap EZ Designs	Available in various reaction packs

1 View .bed files using Roche SignalMap software (available at *sequencing.roche.com/products/software.html* or the Internet-based UCSC Genome browser).

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15 to +25 °C) unless indicated otherwise.
- Unless otherwise specified, all mixing steps are listed as 'mix thoroughly' and indicate that the sample should be combined by either vortexing for 10 seconds or pipetting up and down 10 times.
- If liquid has collected in a tube's cap after mixing, gently tap or briefly spin the sample to collect the liquid into the tube's bottom, ensuring that the mixture remains homogeneous before progressing to the next step.
- It is recommended to perform thermocycler incubations using a thermocycler with a heated lid set to track 10 °C above the block temperature.

Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for use with the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier	Catalog No.
DNA Vacuum Concentrator (1.5 mL tubes) (optional)	*Multiple Vendors*	
Covaris Ultra Sonicator (optional)	Covaris	Multiple models (*e.g.* S220, E220)
DynaMag-2 Magnet (16 x 2.0 mL tube holder) (optional)	Thermo Fisher	12321D
DynaMag-96 Side Magnet	Thermo Fisher	12331D
Microcentrifuge (16,000 x g capability)	*Multiple Vendors*	
Spectrophotometer	NanoDrop	ND-1000
Bioanalyzer 2100	Agilent	
LightCycler® 480 Instrument II (optional, *Appendix A*)	Roche	05 015 243 001 (384-well) -or- 05 015 278 001 (96-well)
Thermocycler (capable of maintaining +47 °C for 16 - 20 hours; programmable heated lid recommended)	*Multiple Vendors*	
Vortex mixer	*Multiple Vendors*	

Consumables Available from Roche

The package sizes listed provide sufficient material to perform a minimum of 24 Sequence Capture experiments.

Component	Package Size/Contents	Catalog No.
LightCycler® 480 Multiwell Plate 384 (with sealing foils) (optional)	5 x 10 plates	04 729 749 001
LightCycler® 480 SYBR Green I Master (2X Mix) (optional)	5 x 1 mL	04 707 516 001
SeqCap Adapter Kit A 96	96 reactions	07 141 530 001
SeqCap Adapter Kit B 96	96 reactions	07 141 548 001
KAPA Dual-Indexed Adapter Set (15 µM)	96 adapters x 20 µL each	08 278 555 702
HyperCap Bead Kit	24 reactions 96 reactions	08 286 418 001 08 286 400 001
HyperCap Target Enrichment Kit	24 reactions 96 reactions	08 286 370 001 08 286 345 001

Library Preparation Kits

Component	Package Size/Contents	Roche Catalog No.	KAPA Catalog No.
KAPA Hyper Prep Kit	8 reactions 24 reactions 96 reactions	07 962 312 001 07 962 347 001 07 962 363 001	KK8500 KK8502 KK8504
KAPA HyperPlus Library Preparation Kit	8 reactions 24 reactions 96 reactions	07 962 380 001 07 962 401 001 07 962 428 001	KK8510 KK8512 KK8514
Confirm availability of ordering kits by Roche part number.			

Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
Agencourt AMPure XP Beads	Beckman Coulter	5 mL 60 mL 450 mL	A63880 A63881 A63882
Agilent DNA 1000 Kit	Agilent	1 kit	5067-1504
Elution buffer (10 mM Tris-HCl, pH 8.0)	*Multiple Vendors*		
Ethanol, 200 proof (absolute), for molecular biology	Sigma-Aldrich	500 mL	E7023-500ML
microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm (25) (required only if using KAPA Hyper Prep Kit)	Covaris, Inc.	1 package of 25 tubes	520045
TE Buffer, 1 X Solution pH 8.0, Low EDTA (required only if using KAPA Hyper Prep Kit)	USB Corporation	100 mL	75793
Tubes: ■ 0.2 mL PCR tubes ■ 1.5 mL microcentrifuge tubes (optional)	*Multiple Vendors*		
Water, PCR Grade	*Sigma-Aldrich*	1 x 25 mL 25 x 1 mL 4 x 25 mL	3315959001 3315932001 3315843001

Use nuclease-free, PCR-grade water for all described protocol steps. Working with a liquid handler system may require a considerably greater excess volume.

Agencourt AMPure XP Beads for library preparation steps must be purchased separately.

Custom Oligonucleotides Purchased from Other Vendors

Component	Concentration	Sequence	Note(s)
qPCR NSC-0237, forward, Oligo	2 µM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'	These oligos are used in qPCR analysis of enrichment (*Appendix A*)
qPCR NSC-0237, reverse, Oligo	2 µM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'	
qPCR NSC-0247, forward, Oligo	2 µM	5' - CCC ACC GCC TTC GAC AT - 3'	
qPCR NSC-0247, reverse, Oligo	2 µM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'	
qPCR NSC-0268, forward, Oligo	2 µM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'	
qPCR NSC-0268, reverse, Oligo	2 µM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'	
qPCR NSC-0272, forward, Oligo	2 µM	5' - CAG CCC CAG CTC AGG TAC AG - 3'	
qPCR NSC-0272, reverse, Oligo	2 µM	5' – ATG ATG CGA GTG CTG ATG ATG - 3'	

Chapter 2. Store the HyperCap Reagents

Chapter 2 describes the storage conditions for the following kits:

- SeqCap EZ, SeqCap EZ Prime, or SeqCap EZ MedExome Probes
- KAPA Hyper Prep Kit or KAPA HyperPlus Library Preparation Kit
- SeqCap Adapter Kit (A and/or B)
- KAPA Dual-Indexed Adapter Kit
- HyperCap Bead Kit
- HyperCap Target Enrichment Kit

Step 1. Aliquot the SeqCap EZ Probe Pool

Upon receipt of the SeqCap EZ, SeqCap EZ Prime, or SeqCap EZ MedExome Probes, undertake the following steps to ensure the highest performance of the SeqCap EZ probe pool to avoid multiple freeze/thaw cycles or potential accidental contamination:

1. If frozen, thaw the tube of SeqCap EZ probe pool on ice.
2. Vortex the SeqCap EZ probe pool for 3 seconds.
3. Centrifuge the tube of SeqCap EZ probe pool at 10,000 x g for 30 seconds to ensure that the liquid is at the bottom of the tube before opening the tube.
4. Aliquot the SeqCap EZ probe pool into single-use aliquots (4.5 µL/aliquot) in 0.2 mL PCR tubes and store at -15 to -25 °C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.
5. When ready to perform the experiment, thaw the required number of single-use SeqCap EZ probe pool aliquots on ice.

The SeqCap EZ probe pool should not undergo multiple freeze/thaw cycles. To help ensure the highest performance of the SeqCap EZ probe pool, Roche recommends aliquoting the SeqCap EZ probe pool into single-use volumes to prevent damage from successive freeze/thaw cycles.

Step 2. Store the Frozen Reagents

Upon receipt, store the KAPA Hyper Prep or KAPA HyperPlus Library Preparation Kit, SeqCap Adapter Kits, KAPA Dual-Indexed Adapter Kit, and HyperCap Target Enrichment Kit, at -15 to -25 °C until use to ensure the highest performance.

Step 3. Store the Refrigerated Reagents

Upon receipt, store the HyperCap Bead kit at +2 to +8 °C until use to ensure the highest performance.

The HyperCap Bead kit must not be frozen.

Chapter 3. Prepare the Sample Library

Chapter 3 describes the two sample library preparation methods. If mechanically fragmenting the gDNA with a Covaris focused-ultrasonicator, use the KAPA Hyper Prep Kit. The KAPA HyperPlus Library Preparation Kit is used for enzymatically fragmenting gDNA. This chapter requires use of components from the following kits:

- KAPA Hyper Prep Kit or KAPA HyperPlus Library Preparation Kit
- SeqCap Adapter Kit (A and/or B)
- KAPA Dual-Indexed Adapter Kit

Ensure that the following are available:

- TE buffer, 1X solution pH 8.0, low EDTA (KAPA Hyper Prep Kit, only)
- Additional PCR-grade water for sample library preparation
- Freshly-prepared 80% ethanol
- Elution buffer (10 mM Tris-HCl, pH 8.0)
- Agencourt AMPure XP Beads (equilibrated to room temperature and fully resuspended prior to use)

References

- *Covaris Focused-ultrasonicator User's Guide*

Sample Requirements

This workflow was validated with 100 ng of high quality gDNA for sample library preparation. Lower input amounts and sample quality may not yield equivalent results. For guidance on lower input amounts or sample quality, contact technical support.

Step 1. Resuspend the SeqCap Adapters (if using single-index adapters)

Resuspension of the SeqCap Adapters must be performed on ice. Lyophilized pellets should be centrifuged prior to resuspension to collect contents at the bottom of the tube, and care should be taken when opening tubes to avoid loss of the lyophilized pellet.

1. Spin the lyophilized SeqCap adapters, contained in the SeqCap Adapter Kit A and/or B, briefly to allow the contents to pellet at the bottom of the tube.
2. Add 50 µL cold, PCR-grade water to each of the 12 tubes labeled 'SeqCap Index Adapter' in the SeqCap Adapter Kit A and/or B. Keep adapters on ice.
3. Briefly vortex the index adapters plus PCR-grade water and spin down the resuspended index adapter tubes.
4. The resuspended index adapter tubes should be stored at -15 to -25 °C.

Step 2. Prepare the Sample Library

The sample library may be prepared following one of two workflows. Choose the instructions for one or the other.

- KAPA Hyper Prep Kit – mechanical fragmentation
- KAPA HyperPlus Library Preparation Kit – enzymatic fragmentation

KAPA HyperPlus Library Preparation Kit

Make sure AMPure XP Beads are removed from storage to allow time for proper equilibration to room temperature.

When assembling a master mix for processing multiple samples, always prepare an appropriate excess (5 to 10% for manual workflows and ~20% for automated liquid handling).

Enzymatic fragmentation is very sensitive to the presence of EDTA, which must be removed or neutralized prior to fragmentation.

Performing a column or bead based purification will remove EDTA present in your samples prior to enzymatic fragmentation. If performing this activity it is best to resuspend or elute using 10 mM Tris-HCl, pH 8.0. Contact Roche Technical Support for assistance with gDNA sample clean-up.

1. Dilute 100 ng of gDNA to be used for library construction in 10 mM Tris –HCl (pH 8.0) to total volume of 35 µL into a 0.2 mL tube or well of PCR plate.
2. Assemble each fragmentation reaction on ice by adding the components in the order shown:

Component	Volume
100 ng gDNA	35 µL
KAPA Frag Buffer (10x)	5 µL
KAPA Frag Enzyme	10 µL
Total	**50 µL**

It is critical that samples be assembled and remain on ice before and after placement in a pre-cooled thermocycler.

3. Mix Fragmentation Reaction thoroughly.
4. Place in a pre-cooled thermocycler, set to instant incubate at 4 °C. Then incubate the samples using the program outlined below:
    1. Step 1: 20 minutes at +37 °C
    2. Step 2: Hold at +4 °C

Fragmentation times may vary due to differences in lab consumables, equipment and DNA quality. Incubation time at 37 °C should be optimized based on individual conditions. For additional guidance, contact Roche Technical Support.

5. Transfer reaction to ice and proceed immediately to the next step.

6. Perform End Repair and A-tailing Reaction as follows:

   a. Prepare a master mix of the following reagents:

End Repair Master Mix	Per Individual Sample Library
KAPA End Repair & A-Tailing Buffer	7 µL
KAPA End Repair & A-Tailing Enzyme Mix	3 µL
Total	**10 µL**

   b. To each 50 µL fragmented sample add 10 µL of End Repair and A-tailing Master Mix.

   c. Mix the End Repair and A-tailing reaction thoroughly.

   d. Place on ice and immediately proceed to next step.

   e. Perform the End Repair and A-Tailing incubation in a thermocycler using the following program with heated lid:

      1. Step 1: 30 minutes at +65 °C
      2. Step 2: Hold at +4 °C

   f. Following the 30 minute incubation, proceed immediately to the next step.

7. Proceed with the Adapter Ligation Reaction Setup:

   a. Prepare a master mix of the following reagents:

Ligation Master Mix	Per Individual Sample Library
PCR-grade water	5 µL
KAPA Ligation Buffer	30 µL
KAPA DNA Ligase	10 µL
Total	**45 µL**

   b. Add Library Adapter (with the desired Index) to the sample well containing the End Repair and A-tailing mix plus DNA.

      - If using the SeqCap Adapters: Add 5 µL of the SeqCap Library Adapter to the sample well.
      - If using the KAPA Dual-Indexed Adapters: Add 5 µL of the KAPA Dual-Indexed Adapter (15 µM) to the sample well.

      Ensure that you record the index used for each sample.

   c. To each sample well that contains 65 µL End Repair and A-tailing mix/DNA/adapter, add 45 µL of the Ligation Master Mix, resulting in a total volume of 110 µL.

   d. Mix the Ligation reaction thoroughly.

   e. Incubate the Ligation reaction at +20 °C for 15 minutes.

   f. Following the incubation, proceed immediately to the next step.

8. Perform the Post-Ligation Cleanup as follows:

   a. To each Ligation Reaction, add 88 µL room temperature AMPure XP Beads that have been thoroughly resuspended.

First Post Ligation Cleanup	Per Individual Sample Library
Ligation Reaction	110 µL
AMPure XP Beads	88 µL
Total	**198 µL**

   b. Mix the Ligation Reaction product and AMPure XP Beads thoroughly.

It is important at this step to ensure that the solution is thoroughly mixed. Insufficient mixing may compromise recovery.

   c. Incubate the samples at room temperature for 5 minutes to allow the DNA to bind to the beads.

   d. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.

   e. Carefully remove and discard the supernatant.

   f. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.

   g. Incubate the samples at room temperature for ≥30 seconds.

   h. Carefully remove and discard the ethanol.

   i. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.

   j. Incubate the samples at room temperature for ≥30 seconds.

   k. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

   l. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

Caution: Over-drying the beads may result in dramatic yield loss.

   m. Remove the samples from the magnetic particle collector.

   n. Thoroughly resuspend the beads in 53 µL of elution buffer (10 mM Tris-HCl, pH 8.0).

   o. Incubate the samples at room temperature for 2 minutes to allow the DNA to elute off the beads.

   p. Place the samples on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.

   q. Transfer 50 µL supernatant to a fresh tube/well.

   r. Proceed immediately to Step 3. Perform the Double-sided size selection.

KAPA Hyper Prep Kit

Make sure AMPure XP Beads are removed from storage to allow time for proper equilibration to room temperature.

When assembling a master mix for processing multiple samples, always prepare an appropriate excess (5 to 10% for manual workflows and ~20% for automated liquid handling).

1. Pipette 100 ng of the gDNA sample of interest into a 0.2 mL tube.
2. Adjust the volume to a total of 52.5 µL using 1x TE buffer (low EDTA) and transfer to a Covaris microTUBE for fragmentation.
3. Fragment the gDNA so that the average DNA fragment size is 180 – 220 bp. Consult manufacturer's instructions for appropriate parameters to achieve this size distribution.
4. Following fragmentation, proceed with the End Repair and A-tailing Reaction Setup:
   a. Transfer 50 µL of the fragmented DNA to a 0.2 mL PCR tube or well of a PCR plate.
   b. Prepare a master mix of the following reagents:

End Repair and A-tailing Master Mix	Per Individual Sample Library
KAPA End Repair & A-Tailing Buffer	7 µL
KAPA End Repair & A-Tailing Enzyme Mix	3 µL
Total	**10 µL**

   c. To each 50 µL fragmented DNA sample, add 10 µL of End Repair and A-tailing Master Mix, to obtain a total volume of 60 µL.
   d. Mix the End Repair and A-tailing reaction thoroughly.
   e. Place on ice and immediately proceed to next step.
   f. Perform the End Repair and A-Tailing incubation in a thermocycler using the following program with heated lid:
      1. Step 1: 30 minutes at +20 °C
      2. Step 2: 30 minutes at +65 °C
      3. Step 3: Hold at +4 °C
   g. Following the 60 minute incubation, proceed immediately to the next step.
5. Perform the Adapter Ligation Reaction as follows::
   a. Prepare a master mix of the following reagents:

Ligation Master Mix	Per Individual Sample Library
PCR-grade water	5 µL
KAPA Ligation Buffer	30 µL
KAPA DNA Ligase	10 µL
Total	**45 µL**

b. Add Library Adapter (with the desired Index) to the sample well containing the End Repair and A-tailing mix plus DNA.

- If using the SeqCap Adapters: Add 5 µL of the SeqCap Library Adapter to the sample well.
- If using the KAPA Dual-Indexed Adapters: Add 5 µL of the KAPA Dual-Indexed Adapter (15 µM) to the sample well.

Ensure that you record the index used for each sample. Centrifuge the KAPA Dual-Indexed Adapter plate before use to ensure that all liquid is at the bottom of the plate wells.

c. To each sample that contains 65 µL End Repair and A-tailing mix/DNA/adapter add 45 µL of the Ligation Master Mix, resulting in a total volume of 110 µL.

d. Mix the Ligation reaction thoroughly.

e. Incubate the Ligation reaction at +20 °C for 15 minutes.

f. Following the incubation, proceed immediately to the next step.

6. Perform the Post-Ligation Cleanup as follows:

a. To each Ligation Reaction product add 88 µL room temperature AMPure XP Beads that have been thoroughly resuspended.

First Post Ligation Cleanup	Per Individual Sample Library
Ligation Reaction product	110 µL
AMPure XP Beads	88 µL
Total	**198 µL**

b. Mix the Ligation Reaction product and AMPure XP Beads thoroughly.

It is important at this step to ensure that the solution is thoroughly mixed. Insufficient mixing may compromise recovery.

c. Incubate the sample at room temperature for 5 minutes to allow the DNA to bind to the beads.

d. Place the sample on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.

e. Carefully remove and discard the supernatant.

f. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.

g. Incubate the sample at room temperature for ≥30 seconds.

h. Carefully remove and discard the ethanol.

i. Keeping the sample on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.

j. Incubate the sample at room temperature for ≥30 seconds.

k. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

l. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

Caution: Over-drying the beads may result in dramatic library loss.

m. Remove the sample from the magnetic particle collector.

n. Thoroughly resuspend the beads in 53 µL of elution buffer (10 mM Tris-HCl, pH 8.0).

o. Incubate the sample at room temperature for 2 minutes to allow the DNA to elute off the beads.

p. Place the sample on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.

q. Transfer 50 µL supernatant to a fresh tube/well.

r. Proceed immediately to Step 3. Perform the Double-Sided Size Selection.

Step 3. Perform the Double-Sided Size Selection

The AMPure XP Beads volume described in this section are recommendations. The resulting library size distributions are dependent upon variables such as the initial fragment sizes going into selection. This procedure may be optimized based on the customer's objectives. If additional guidance is needed, contact Roche Technical Support.

1. To each sample containing 50 µL supernatant, add 35 µL room temperature AMPure XP Beads that have been thoroughly resuspended for total volume of 85 µL.

Double-Sided Size Selection	Per Individual Sample Library
Resuspended DNA	50 µL
AMPure XP Beads	35 µL
Total	**85 µL**

2. Mix thoroughly.
3. Incubate the sample at room temperature for 5 minutes to allow library fragments larger than ~450 bp to bind to the beads.
4. Place the samples on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
5. Carefully transfer 80 µL of the supernatant containing library fragments smaller than ~450 bp to a new tube/well.

Do NOT discard the supernatant at this step. It is also critical to not transfer any beads with the supernatant.

6. To the 80 µL supernatant add 10 µL of room temperature Agencourt AMPure XP Beads.
7. Thoroughly resuspend the beads by pipetting up and down multiple times.
8. Incubate the sample at room temperature for 5 minutes to allow library fragments larger than ~250 bp to bind to the beads.
9. Place the samples on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
10. Carefully remove and discard most of the supernatant without disturbing beads.

11. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.
12. Incubate the sample at room temperature for ≥30 seconds.
13. Carefully remove and discard the ethanol.
14. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.
15. Incubate the sample at room temperature for ≥30 seconds.
16. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
17. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

    Caution: Over-drying the beads may result in dramatic yield loss.
18. Remove the samples from the magnetic particle collector.
19. Thoroughly resuspend the beads in 23 µL of elution buffer (10 mM Tris-HCl, pH 8.0) or PCR-grade water.
20. Incubate the sample at room temperature for 2 minutes to allow the DNA to elute off the beads.
21. Place the samples on a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
22. Transfer the clear supernatant (~20 µL) to a new tube/well and proceed with the amplification of the sample library as detailed in *Chapter 4*.

Chapter 4. Amplify the Sample Library Using LM-PCR

This chapter describes how to amplify the sample library (prepared in*Chapter 3*) using LM-PCR in preparation for hybridization to the SeqCap EZ probe pool. This chapter requires the use of the components from the following kits:

- KAPA Hyper Prep Kit or KAPA HyperPlus Library Preparation Kit

Ensure that the following is available:

- Agencourt AMPure XP Beads
- Freshly-prepared 80% ethanol
- Elution Buffer (10 mM Tris-HCl, pH 8.0)

References

- *Thermocycler Manual*
- *NanoDrop ND-1000 Manual*
- *Agilent DNA 1000 Kit Guide*

Sample Requirements

For each sample library to be captured, 20 µL of the sample library from *Chapter 3* is amplified via Pre-Capture LM-PCR.

Step 1. Prepare the Pre-Capture LM-PCR Master Mix

We recommend the inclusion of negative (water) and positive (previously amplified library) controls in the Pre-Capture LM-PCR step.

Instructions for preparing an individual PCR reaction are shown here. When assembling a master mix for processing multiple samples, always prepare an appropriate excess (5 to 10% for manual workflows and ~20% for automated liquid handling).

1. Prepare a master mix of the following reagents:

Pre-Capture LM-PCR Master Mix	Per Individual Sample Library or Negative Control
KAPA HiFi HotStart ReadyMix (2X)*	25 µL
Library Amplification Primer Mix (10X)*	5 µL
Total	**30 µL**

* Note: The Library Amplification Primer Mix (10X) and KAPA HiFi HotStart ReadyMix (2X) are contained within the KAPA Hyper Prep or KAPA HyperPlus Library Preparation Kit.

2. To each sample library in a 0.2 mL PCR tube or well of a PCR plate and control samples, add 30 µL of Pre-Capture LM-PCR Master Mix.
3. Mix thoroughly.

Step 2. Perform the Pre-Capture PCR Amplification

1. Place the samples in the thermocycler.

   It is recommended to set the heated lid of the thermocycler to track +10 °C above the incubation temperature during amplification steps.

2. Amplify the sample library using the following Pre-Capture LM-PCR program:

   - Step 1: 45 seconds at +98 °C
   - Step 2: 15 seconds at +98 °C
   - Step 3: 30 seconds at +60 °C
   - Step 4: 30 seconds at +72 °C
   - Step 5: Repeat as directed below for the library preparation kit used:

Library Preparation Kit	Adapter Type	Go to Step 2:
KAPA Hyper Prep Kit	SeqCap Adapters	8 times (9 total cycles)
	KAPA Dual-Indexed Adapters	10 times (11 total cycles)
KAPA HyperPlus Library Preparation Kit	SeqCap Adapters	6 times (7 total cycles)
	KAPA Dual-Indexed Adapters	6 times (7 total cycles)

   - Step 6: 1 minute at +72 °C
   - Step 7: Hold at +4 °C

3. Store the reaction at +2 to +8 °C until ready for cleanup, for up to 72 hours.

Step 3. Purify the Amplified Sample Library using Agencourt AMPure XP Beads

1. Allow the Agencourt AMPure XP Beads to warm to room temperature for at least 30 minutes before use.
2. Vortex the AMPure XP Beads for 10 seconds before use to ensure a homogeneous mixture of beads.
3. Add 90 µL AMPure XP Beads to the 50 µL amplified sample library.
4. Vortex for 10 seconds.
5. Incubate at room temperature for 5 minutes to allow the DNA to bind to the beads.
6. Place the samples containing the bead-bound DNA in a magnetic particle collector. Allow the solution to clear.
7. Once clear, remove and discard the supernatant being careful not to disturb the beads.
8. Add 200 µL freshly-prepared 80% ethanol to the tube/wells containing the bead-bound DNA samples. The samples should be left in the magnetic particle collector during this step.
9. Incubate at room temperature for ≥30 seconds.
10. Remove and discard the 80% ethanol.
11. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.
12. Incubate the samples at room temperature for ≥30 seconds.

13. Remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
14. Allow the beads to dry at room temperature with the tube lid open for 5 minutes (or until dry).

    Over drying of the beads can result in yield loss.

15. Remove the samples from the magnetic particle collector.
16. Resuspend the DNA using 53 µL of 10 mM Tris-HCl, pH 8.0 or PCR-grade water.

    It is critical that each amplified sample library is eluted with PCR-grade water and not buffer EB or 1X TE if using a DNA vacuum concentrator to prepare hybridization as described in *Appendix B*.

17. Vortex for 10 seconds to mix to ensure that all of the beads are resuspended.
18. Incubate at room temperature for 2 minutes.
19. Place the samples back in the magnetic particle collector and allow the solution to clear.
20. Remove 50 µL of the supernatant that now contains the amplified sample library and transfer into a new tube/well.
21. Purified, amplified libraries can be stored at 2 to 8 °C for 1-2 weeks or at -15 to -25 °C.

Step 4. Check the Quality of the Amplified Sample Library

1. Measure the A260/A280 ratio of the amplified sample library to quantify the DNA concentration using a NanoDrop spectrophotometer and determine the DNA quality.

   When working with samples that will be pooled for hybridization (*i.e.* multiplex Sequence Capture), accurate quantitation is **essential**. Slight differences in the mass of each sample combined to form the 'Multiplex DNA Sample Library Pool' will result in variations in the total number of sequencing reads obtained for each sample in the library pool.

   - The A_{260}/A_{280} ratio should be 1.7 to 2.0.
   - The sample library yield should be ≥ 1.0 µg.
   - The negative control yield should be negligible. If this is not the case, the measurement may be high due to the presence of unincorporated primers carried over from the LM-PCR reaction and not an indication of possible contamination between amplified sample libraries.

2. Analyze 1 µL of each amplified sample library (and any controls) using an Agilent Bioanalyzer DNA 1000 assay according to manufacturer's instructions.

   - Pre-capture libraries should display a fragment size distribution in the range of 150 to 500 bp, with a peak at ~320 bp (*Figure 2*, *Figure 3*). The negative control should not show any significant signal within this size range, which could indicate contamination between amplified sample libraries. Sharp peaks may be visible in the region <150 bp. These peaks correspond to unincorporated primers, primer-dimers or carry-over adapter-dimer and will not interfere with the capture process.

- The negative control should not show any signal above baseline within the 150 to 400 bp size range, which could indicate contamination between amplified sample libraries, but it may exhibit sharp peaks visible below 150 bp. If the negative control reaction shows a positive signal by the NanoDrop spectrophotometer, but the Bioanalyzer trace indicates only the presence of a sharp peak below 150 bp in size, then the negative control should not be considered contaminated.

3. If the amplified sample library meets these requirements, proceed to*Chapter 5*. If the amplified sample library does not meet these requirements, reconstruct the library.

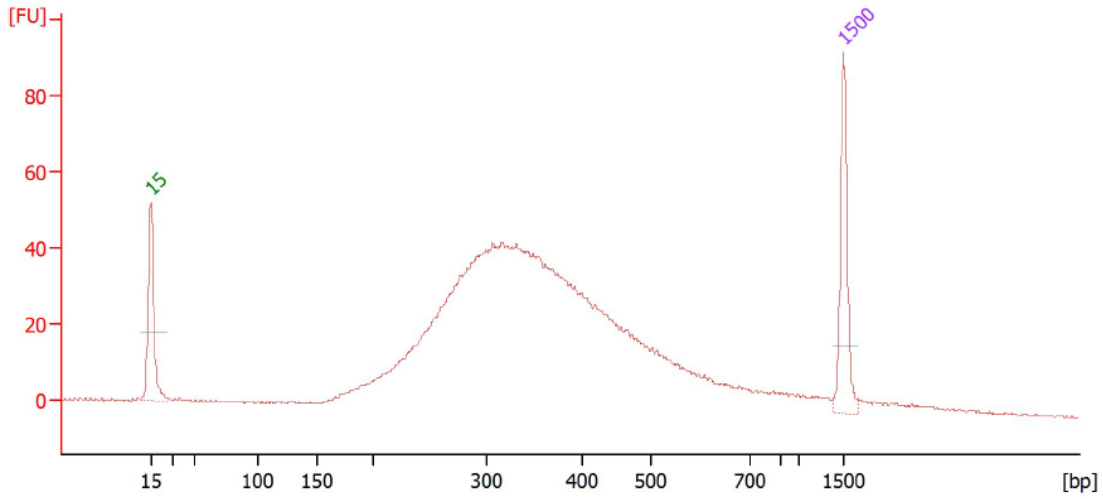


Figure 2: Example of an amplified HyperPlus sample library analyzed using an Agilent Bioanalyzer DNA 1000 assay

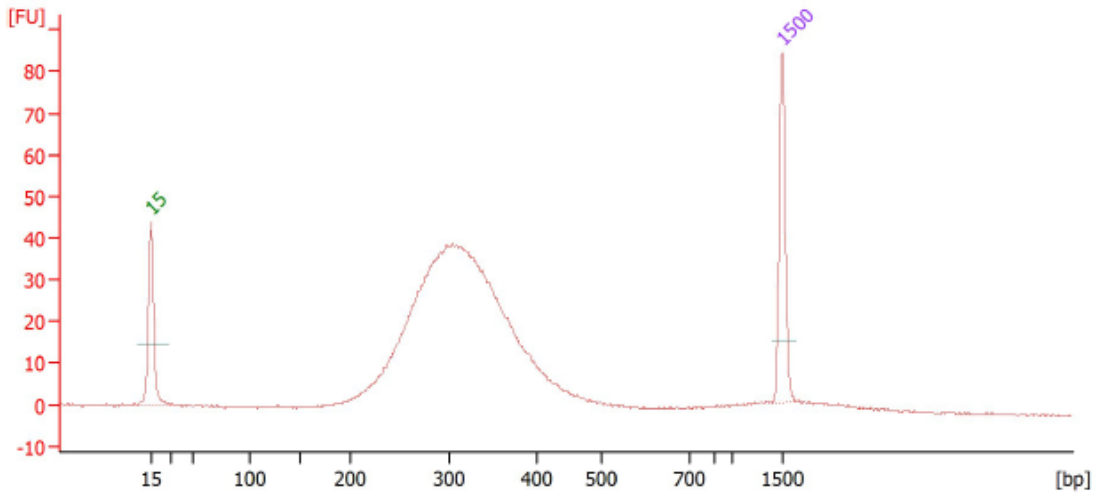


Figure 3: Example of an amplified Hyper Prep sample library analyzed using an Agilent Bioanalyzer DNA 1000 assay

Chapter 5. Hybridize the Sample and SeqCap EZ Probe Pool

Chapter 5 describes the Roche protocol for hybridization of the amplified sample libraries and the SeqCap EZ probe pool. This chapter requires the use of components from the following kits:

- SeqCap EZ probe pool (refers to SeqCap EZ, SeqCap EZ Prime, or SeqCap EZ probes)
- HyperCap Target Enrichment Kit
- HyperCap Bead Kit

Ensure that the following is available:

- Freshly-prepared 80% ethanol

The hybridization protocol requires a thermocycler capable of maintaining +47 °C for 16 to 20 hours. A programmable heated lid is required.

Note: In this chapter we use the term 'Multiplex DNA Sample Library Pool', however a single DNA sample library may be captured using the same instructions. It is not required to capture more than one library at a time.

Step 1. Prepare for Hybridization

1. Remove the appropriate number of 4.5 µL SeqCap EZ probe pool aliquots (one per hybridization) from the -15 to -25 °C freezer and allow them to thaw on ice.

Step 2. Prepare the Multiplex DNA Sample Library Pool

1. Thaw on ice each of the uniquely indexed amplified DNA sample libraries that will be included in the multiplex capture experiment (generated in *Chapter 4*).
2. Mix together equal amounts (by mass) of each of these amplified DNA sample libraries to obtain a single pool with a combined mass of 1 µg. This mixture will subsequently be referred to as the 'Multiplex DNA Sample Library Pool'. If you plan on performing the optional measurement of enrichment using qPCR (*Appendix A*), set aside equal amounts (by mass) of each of these amplified DNA sample libraries to obtain a single pool with a combined mass of at least 1.25 µg. One µg of the multiplex DNA sample library pool will be used in the sequence capture hybridization step and remainder will be used for measurement of enrichment using qPCR (*Appendix A*).

To obtain equal numbers of sequencing reads from each component libraries in the Multiplex DNA Sample Library Pool upon completion of the experiment, it is very important to combine identical amounts of each independently amplified DNA sample library at this step. Accurate quantification and pipetting are critical.

Note: Store remaining Multiplex DNA Sample Library Pool at -15 to -25 °C until use in measurement of enrichment using qPCR (*Appendix A*).

Step 3. Prepare the Hybridization Sample

This step outlines how to prepare the hybridization sample using a bead based approach with AMPure XP Beads. Alternatively vacuum centrifugation can be used, see *Appendix B*.

Note: When working with non-human gDNA, consider using the SeqCap EZ Developer Reagent (catalog number 06684335001) in place of COT Human DNA. When using the SeqCap EZ Developer Reagent, add 10 µL of this reagent to each hybridization instead of COT Human DNA.

1. Allow the AMPure XP Beads, contained in the HyperCap Bead Kit, to warm to room temperature for at least 30 minutes before use.
2. Add 5 µL of COT Human DNA (1 mg/mL), contained in the HyperCap Target Enrichment Kit, to a new tube/well.
3. Add 1 µg of Multiplex DNA Sample Library to the sample containing 5 µL of COT Human DNA.
4. Add 5 µL of the HyperCap Universal Blocking Oligos to the Multiplex DNA Sample Library Pool plus COT Human DNA.
5. Determine the total volume of the above mixture by adding input volumes of COT Human DNA, Multiplex DNA Sample Library Pool and HyperCap Universal Blocking Oligos.
6. Add 2 volumes of AMPure XP Beads (equilibrated to room temperature and fully resuspended) to the above mixture.

   For example, if the total calculated volume of COT Human DNA, Multiplex DNA Sample Library Pool and Universal Blocking Oligos from previous step is 60 µL, add 120 µL AMPure XP Beads.

7. Mix thoroughly by vortexing for ten seconds.
8. Let the sample incubate at room temperature for 10 minutes to allow the sample library to bind to the beads.
9. Place the samples on the magnetic particle collector to capture the beads. Allow the solution to clear.
10. Once clear, remove and discard the supernatant being careful not to disturb the beads.
11. Add 190 µL 80% ethanol to the samples containing the bead-bound DNA samples. The samples should be left on the magnetic particle collector during this step.
12. Incubate at room temperature for ≥30 seconds.
13. Carefully remove and discard the 80% ethanol. Try to remove all residual ethanol without disturbing the beads.
14. Allow the beads to dry at room temperature with the tube lid open for 5 minutes (or until dry).
15. Prepare a master mix of the following reagents, scaling up to reflect number of captures:
    - 7.5 µL of 2X Hybridization Buffer
    - 3 µL of Hybridization Component A
16. Add 10.5 µL of the Hybridization Buffer/Hybridization Component A mix from previous step to the bead-bound DNA samples.

17. Remove samples from the magnetic particle collector and mix thoroughly by vortexing. It is important that enough mixing is performed at this step to yield a homogeneous mixture.
18. Let sit at room temperature for 2 minutes.
19. Place samples on a magnetic particle collector.
20. After liquid clears, remove 10.5 µL of supernatant (entire volume) and place in a new tube/well containing 4.5ul of the SeqCap EZ probe pool.

    Users may experience slight bead carryover when transferring the supernatant. This is unlikely to significantly impact results. If you are concerned about the amount of beads left in your sample, contact technical support.
21. Mix thoroughly by vortexing for 10 seconds.
22. Perform the hybridization incubation in a thermocycler using the following program with heated lid set to 10 °C above block temperature:

    - 95 °C for 5 minutes
    - 47 °C for 16 to 20 hours

    For incubation at 47 °C for 16 to 20 hours, it is important that the thermocycler's heated lid is turned on and set to maintain 10 °C above the hybridization temperature (+57 °C). The sample must remain at 47 °C until it is transferred to the capture beads in *Chapter 6*, Step 3.
23. Continue to *Chapter 6* for Washing and Recovery. Do not store the sample prior to washing and recovery.

Chapter 6. Wash and Recover Captured Multiplex DNA Sample

Chapter 6 describes the process for the washing and recovery of the captured multiplex DNA sample from the hybridization of the Multiplex DNA Sample Library Pool and SeqCap EZ probe pool. This chapter requires the use of components from the following kits:

- HyperCap Target Enrichment Kit
- HyperCap Bead Kit

Ensure that the following is available:

- Additional PCR-grade water for buffer preparation and elution

Step 1. Prepare Sequence Capture and Bead Wash Buffers

Volumes for an individual capture are shown here. When preparing 1X buffers for processing multiple reactions, prepare an excess volume of ~5% to allow for complete pipetting (liquid handling systems may require an excess of ~20%).

1. Dilute 10X Wash Buffers (I, II, III and Stringent) and 2.5X Bead Wash Buffer, contained in the SeqCap Hybridization and Wash Kit, to create 1X working solutions. Volumes listed below are sufficient for one capture.

Concentrated Buffer	Volume of Concentrated Buffer	Volume of PCR-grade Water	Total Volume of 1X Buffer*
10X Stringent Wash Buffer	40 µL	360 µL	**400 µL**
10X Wash Buffer I	30 µL	270 µL	**300 µL**
10X Wash Buffer II	20 µL	180 µL	**200 µL**
10X Wash Buffer III	20 µL	180 µL	**200 µL**
2.5X Bead Wash Buffer	100 µL	150 µL	**250 µL**

*Store working solutions at room temperature (+15 to +25 °C) for up to 2 weeks. The volumes in this table are calculated for a single experiment; scale up accordingly if multiple samples are processed.

Step 2. Prepare the Capture Beads

1. Allow the Capture Beads, contained in HyperCap Bead Kit, to equilibrate to room temperature for 30 minutes prior to use.
2. Vortex the capture beads for 15 seconds before use to ensure a homogeneous mixture of beads.
3. Aliquot 50 µL of beads for each capture into a 0.2 mL or 1.5 mL tube (*i.e.* for one capture use 50 µL beads and for four captures use 200 µL beads, *etc.*). Enough beads for two captures and twelve captures can be prepared in a single 0.2 mL tube and 1.5 mL tube, respectively.
4. Place the tubes on a magnetic particle collector. Allow the solution to clear (should take less than 5 minutes).
5. Remove and discard the supernatant being careful not to disturb the beads. Any remaining traces of liquid will be removed with subsequent wash steps.

6. While the tubes are on the magnetic particle collector, add twice the initial volume of beads of 1X Bead Wash Buffer (*i.e.* for one capture use 100 µL of buffer and for four captures use 400 µL buffer, *etc.*).
7. Remove tubes from the magnetic particle collector and mix thoroughly by vortexing.
8. Place the tubes back on the magnetic particle collector to bind the beads.
9. Once clear, remove and discard the liquid.
10. Repeat Steps 2.6 - 2.9 for a total of two washes.
11. After removing the buffer following the second wash, add 1X the initial volume of beads of 1X Bead Wash Buffer (*i.e.* 50 µL buffer per capture).
12. Remove tubes from magnetic particle collector and mix thoroughly by vortexing for 10 seconds.
13. Aliquot 50 µL of resuspended beads into new tube/well for each capture.
14. Place the tubes on magnetic particle collector to bind the beads. Allow the solution to clear.
15. Once clear, remove and discard the supernatant.
16. The Capture Beads are now ready to bind the captured DNA. Proceed immediately to the next step.

Do not allow the Capture Beads to dry out. Small amounts of residual Bead Wash Buffer will not interfere with binding of DNA to the Capture Beads.

Step 3. Bind DNA to the Capture Beads

1. Transfer one hybridization sample to a single prepared tube/well of Capture Beads from the previous step.
2. Mix thoroughly by vortexing for 10 seconds.
3. Bind the captured sample to the beads by placing the samples in a thermocycler set to +47 °C for 15 minutes (heated lid set to +57 °C).

Step 4. Wash the Capture Beads Plus Bead-Bound DNA

1. After the 15-minute incubation, remove the samples from the thermocycler.

Thermocycler should remain at 47 °C (heated lid turned on and set to maintain +57 °C) for following steps.

2. Add 100 µL of 1X Wash Buffer I to the 15 µL of Capture Beads plus bead-bound DNA.
3. Mix thoroughly by vortexing for 10 seconds.
4. Place the samples on a magnetic particle collector to capture the beads. Allow the solution to clear.
5. Once clear, remove and discard the supernatant being careful not to disturb the beads.
6. Add 200 µL of 1X Stringent Wash Buffer to each sample.
7. Remove the samples from the magnetic particle collector.
8. Mix to homogeneity by vortexing for at least 10 seconds.
9. Place on thermocycler pre-heated to +47 °C, close lid (set to +57 °C) and incubate for 5 minutes.

10. After incubating 5 minutes, remove the sample from thermocycler and place on a magnetic particle collector to capture the beads. Allow the solution to clear.
11. Once clear, remove and discard the supernatant being careful not to disturb the beads
12. Repeat Steps 4.6 - 4.11 for a total of two washes using 1X Stringent Wash Buffer.
13. Add 200 µL of 1X Wash Buffer I.
14. Mix thoroughly by vortexing for 10 seconds. Ensure that the mixture is homogeneous.
15. Incubate at room temperature for 1 minute.
16. Place the samples on a magnetic particle collector to capture the beads. Allow the solution to clear.
17. Once clear, remove and discard the supernatant being careful not to disturb the beads.
18. Add 200 µL of 1X Wash Buffer II.
19. Mix thoroughly by vortexing for 10 seconds. Ensure that the mixture is homogeneous.
20. Incubate at room temperature for 1 minute.
21. Place the samples on a magnetic particle collector to capture the beads. Allow the solution to clear.
22. Once clear, remove and discard the supernatant being careful not to disturb the beads.
23. Add 200 µL of 1X Wash Buffer III.
24. Mix thoroughly by vortexing for 10 seconds. Ensure that the mixture is homogeneous.
25. Incubate at room temperature for 1 minute.
26. Place the samples on a magnetic particle collector to capture the beads. Allow the solution to clear.
27. Once clear, remove and discard the supernatant being careful not to disturb the beads.
28. Remove the samples from the magnetic particle collector.
29. Add 15 µL PCR-grade water to each tube/plate well of bead-bound DNA sample.
30. Store the beads plus captured samples at -15 to -25 °C or proceed to *Chapter 7*.

There is no need to elute DNA off the beads. The beads plus captured DNA will be used as template in the LM-PCR as described in *Chapter 7*.

Chapter 7. Amplify Captured Multiplex DNA Sample Using LM-PCR

Chapter 7 describes the amplification of captured Multiplex DNA sample, bound to the Capture Beads, using LM-PCR. One reaction is performed per sample. This chapter requires the use of components from the following kits:

- HyperCap Target Enrichment Kit
- HyperCap Bead Kit

In addition, ensure that the following are available:

- Additional PCR-grade water for 80% ethanol preparation and elution
- Freshly-prepared 80% ethanol
- Elution buffer (10 mM Tris-HCl, pH 8.0), optional

References

- *Thermocycler Manual*
- *Agilent DNA 1000 Kit Guide*

Step 1. Resuspend the Post-LM-PCR Oligos

1. Briefly spin the lyophilized 'Post-LM-PCR Oligos 1 & 2' oligos, contained in the HyperCap Target Enrichment Kit, to allow the contents to pellet at the bottom of the tube. Note that both oligos are contained within a single tube.
2. Add 480 µL PCR-grade water to the tube of centrifuged oligos.
3. Briefly vortex the resuspended oligos.
4. Spin down the tube to collect the contents.
5. The resuspended oligo tube should be stored at -15 to -25 °C.

Step 2. Prepare the Post-Capture LM-PCR Master Mix

Instructions for preparing individual PCR reactions are shown here. When assembling a master mix for processing multiple samples, prepare an excess volume of ~5% to allow for complete pipetting (liquid handling systems may require an excess of ~20%).

1. Prepare a master mix of the following reagents

Post-Capture LM-PCR Master Mix	Per Individual DNA Sample PCR Reaction
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Post-LM-PCR Oligos 1 & 2, 5 µM*	5 µL
Total	**30 µL**

* Note: The post-capture LM-PCR Oligos and the KAPA HiFi HotStart ReadyMix (2X) are contained within the HyperCap Target Enrichment Kit.

2. Add 30 µL Post-Capture LM-PCR Master Mix to 0.2 mL tube or well of PCR plate.
3. Mix thoroughly by vortexing for 10 seconds the bead-bound DNA from *Chapter 6* .
4. Aliquot 20 µL of bead-bound DNA as template into the tube/well with the 30ul Post-capture LM-PCR Master Mix. (If performing a negative control, add 20ul PCR-grade water to this tube/well).

   Total volume of sample with beads will be approximately 20 µL (15 µL of water with bead volume). If volume is <20ul, add more water to achieve this volume.

5. Mix thoroughly by pipetting up and down several times.

Step 3. Perform the Post-Capture PCR Amplification

1. Place the sample in the thermocycler.

   It is recommended to set the heated lid of the thermocycler to track +10 °C above the incubation temperature during amplification steps.

2. Amplify the captured DNA using the following Post-Capture LM-PCR program:
   - Step 1: 45 seconds at +98 °C
   - Step 2: 15 seconds at +98 °C
   - Step 3: 30 seconds at +60 °C
   - Step 4: 30 seconds at +72 °C
   - Step 5: Go to Step 2, repeat 13 times (for a total of 14 cycles)
   - Step 6: 1 minutes at +72 °C
   - Step 7: Hold at +4 °C
3. Store reactions at +2 to +8 °C until ready for purification, up to 72 hours.

Step 4. Purify the Amplified Captured Multiplex DNA Sample using AMPure XP Beads

1. Allow the AMPure XP Beads, contained in the HyperCap Bead Kit, to warm to room temperature for at least 30 minutes before use.
2. Vortex the AMPure XP Beads for 10 seconds before use to ensure a homogenous mixture of beads.
3. Add 90 µL AMPure XP Beads to the 50 µL amplified captured Multiplex DNA Sample library.
4. Mix thoroughly by vortexing for 10 seconds.
5. Incubate at room temperature for 5 minutes to allow the captured sample library to bind to the beads.
6. Place the samples containing the bead-bound DNA on a magnetic particle collector to capture the beads. Allow the solution to clear.
7. Once clear, remove and discard the supernatant being careful not to disturb the beads.
8. Add 200 µL freshly-prepared 80% ethanol to the samples containing the beads plus sample library. The samples should be left in the magnetic particle collector during this step.
9. Incubate at room temperature for ≥30 seconds.
10. Remove and discard the 80% ethanol.
11. Keeping the samples on the magnetic particle collector, add 200 µL of freshly-prepared 80% ethanol.
12. Incubate the samples at room temperature for ≥30 seconds.
13. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
14. Allow the beads to dry at room temperature with the tube lid open for 5 minutes (or until dry).

    Over drying of the beads can result in yield loss.
15. Remove the samples from the magnetic particle collector.
16. Resuspend the DNA using 53 µL of 10 mM Tris-HCl, pH 8.0 or PCR-grade water.
17. Vortex at least 10 seconds to ensure that all of the beads are resuspended.
18. Incubate at room temperature for 2 minutes.
19. Place the samples back on the magnetic particle collector and allow the solution to clear.
20. Remove 50 µL of the supernatant that now contains the amplified captured Multiplex DNA Sample Library Pool and transfer into a new tube/well.

Step 5. Determine the Concentration, Size Distribution, and Quality of the Amplified Captured Multiplex DNA Sample

1. Quantify the DNA concentration and measure the A260/A280 ratio of the amplified captured multiplex DNA and negative control using a NanoDrop spectrophotometer.

   - The A_{260}/A_{280} ratio should be 1.7 to 2.0.
   - The LM-PCR yield should be 500 ng.
   - The negative control should not show significant amplification, which could be indicative of contamination.

   If the amplified captured multiplex DNA does not meet the A260/A280 ratio requirement, purify again using the Agencourt AMPure XP Beads.

2. Run 1 µL of the amplified captured multiplex DNA sample and negative control using an Agilent Bioanalyzer DNA 1000 chip. Run the chip according to manufacturer's instructions. Amplified captured multiplex DNA should exhibit the following characteristics:

   - The average fragment length should be between 150 to 500 bp.

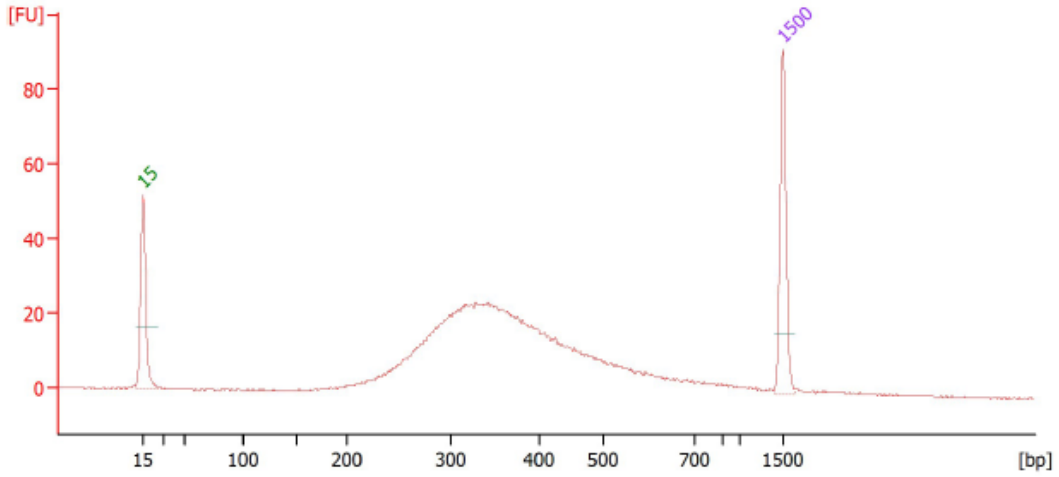


Figure 4: Example of successfully amplified captured multiplex DNA using HyperPlus sample library analyzed using an Agilent Bioanalyzer DNA 1000 chip

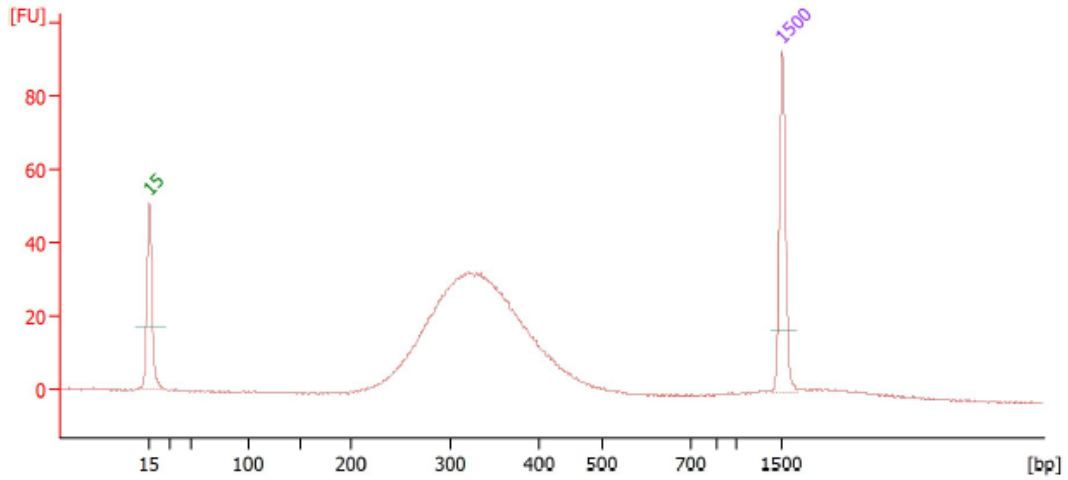


Figure 5: Example of successfully amplified captured multiplex DNA using Hyper Prep sample library analyzed using an Agilent Bioanalyzer DNA 1000 chip

3. The amplified captured multiplex DNA is ready for sequencing. If you would like to check enrichment performance prior to sequencing, conduct qPCR as described in *Appendix A*.

Appendix A. Measure Enrichment Using qPCR

Appendix A describes the qPCR assays employed as internal quality controls for Roche SeqCap experiments performed with human total gDNA. If you are a first time user or have concerns about successful completion of the workflow, this Appendix will help you determine the level of enrichment obtained from your sample. These assays estimate the relative fold enrichment by measuring the relative abundance of control targets in the pre- and post-capture LM-PCR reactions. The genomic loci recognized by these assays are included as capture targets in every SeqCap EZ probe pool and provide an inexpensive way to determine whether the capture was successful prior to sequencing.

This chapter requires the use of the following reagents:

- LightCycler® 480 SYBR Green I Master (2x Mix)
- Additional PCR-grade water
- Custom Oligonucleotides:

Component	Concentration	Sequence
qPCR NSC-0237, forward, Oligo	2 µM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'
qPCR NSC-0237, reverse, Oligo	2 µM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'
qPCR NSC-0247, forward, Oligo	2 µM	5' - CCC ACC GCC TTC GAC AT - 3'
qPCR NSC-0247, reverse, Oligo	2 µM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'
qPCR NSC-0268, forward, Oligo	2 µM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'
qPCR NSC-0268, reverse, Oligo	2 µM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'
qPCR NSC-0272, forward, Oligo	2 µM	5' - CAG CCC CAG CTC AGG TAC AG - 3'
qPCR NSC-0272, reverse, Oligo	2 µM	5' – ATG ATG CGA GTG CTG ATG ATG - 3'

References

- *LightCycler® 480 User's Guide*

Perform the qPCR Assay

1. Determine the number of DNA samples to be analyzed:
   - A 'DNA sample' in this chapter is defined as one amplified sample library (*Chapter 4*) and the corresponding amplified captured multiplex DNA (*Chapter 7*).

   If samples are multiplexed pre-capture, you only need one representative pre-capture amplified sample library for the corresponding amplified captured multiplex DNA sample.

   Instructions for preparing an individual PCR reaction are shown here. When assembling a master mix for processing multiple samples, prepare an excess volume of ~5% to allow for complete pipetting (liquid handling systems may require an excess of ~20%).

- Assuming the standard set of four Roche SeqCap EZ (NSC) control locus qPCR assays will be used and qPCR assays will be performed in triplicate, each DNA sample will require 24 individual qPCR reactions for analysis.
- One negative control (*i.e.* no-template-control, NTC) should always be included to monitor for contamination in qPCR assay primers, other qPCR reagents, and the entire qPCR process.
- One positive control template, ideally consisting of the original genomic DNA starting material, should always be included to verify assay function.
- The negative and positive controls will each require twelve additional qPCR reactions (*Figure 6*).

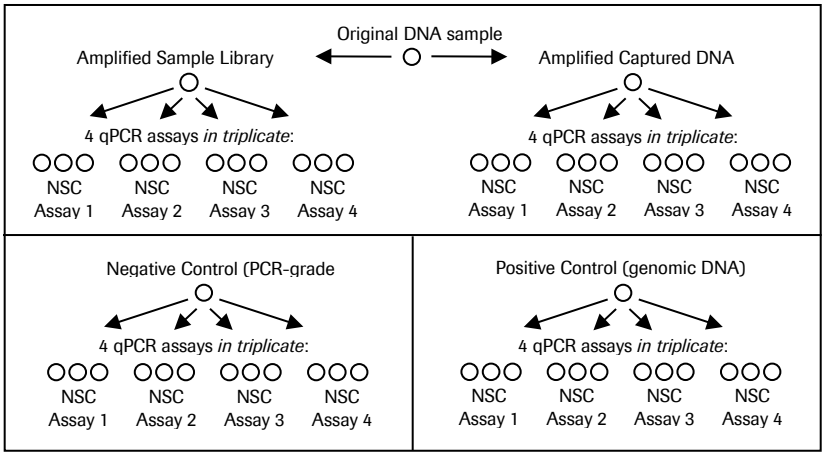


Figure 6: qPCR experimental overview

2. Dilute the following samples to a concentration of 5 ng/µL in 100 µL of PCR-grade water:
   - Genomic DNA (positive control template)
   - Amplified sample library (one representative per multiplexed capture experiment)
   - Each amplified captured multiplex DNA sample
3. Make a master mix for each of the four NSC assays:

qPCR Master Mix	Per Reaction
PCR-grade water	5.9 µL
NSC Assay forward primer (2 µM)	0.3 µL
NSC Assay reverse primer (2 µM)	0.3 µL
SYBR Green Master Mix (2x)	7.5 µL
Total	**14 µL**

Master Mix example: For an experiment that contains a single captured sample, the following numbers of reactions are required (scale-up captured and non-captured reaction numbers based on the number of samples in your particular experiment):

- 3 replicate reactions per NSC Assay (12 total reactions) – captured sample
- 3 replicate reactions per NSC Assay (12 total reactions) – non-captured sample
- 3 replicate reactions per NSC Assay (12 total reactions) – positive control
- 3 replicate reactions per NSC Assay (12 total reactions) – negative control

The template is defined as amplified sample library, amplified captured multiplex DNA, positive control genomic DNA, or negative control PCR-grade water, templates. The final concentration of templates in the reaction should be 0.333 ng/µL, except for the negative control PCR-grade water template.

4. Aliquot 14 µL of each master mix to the appropriate well into a LightCycler® 480 Multiwell Plate 384 and add 1 µL of the appropriate sample:

qPCR Reaction	Per Reaction
NSC qPCR Master Mix	14 µL
Template (pre- or post-capture library, gDNA control, or H2O)	1 µL
Total	**15 µL**

5. Seal the plate with a plate seal.
6. Centrifuge the 384-well plate in a plate centrifuge at 6,000 x g for 10 seconds to collect the reagents at the bottom of the wells.
7. Program the qPCR instrument using the conditions specified in *Table 1*.

These conditions are optimized for use with the LightCycler® 480 Instrument II and LightCycler® 480 SYBR Green I 2X Master Mix. The use of a different thermocycler or reagents could require altering these conditions to achieve optimal results.

Program Name	Cycles	Analysis Mode	Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Acquisition Mode
Pre-incubation	1	None	95	00:10:00	4.8	---	None
Amplification	40	Quantification	95	00:00:10	4.8	---	None
			60	00:01:00	2.5	---	Single
Melting Curve	1	Melting Curves	95	00:00:10	4.8	---	None
			65	00:01:00	2.5	---	None
			95	---	---	5	Continuous
Cooling	1	None	40	00:00:10	2	---	None

Table 1: qPCR instrument cycling conditions

8. Following the completion of the qPCR program, run the Absolute Quantification Analysis Module within the LightCycler® 480 Software using the 2nd Derivative Maximum Method.
9. Perform a melting curve (dissociation) analysis to verify that nonspecific amplification products, primer dimers, and other artifacts are not contributing to the Cp values for any samples.
10. Copy or export the Cp values to a spreadsheet program for further analysis.

Cp (crossing point) values reported by the LightCycler® 480 Instrument II and software are analogous to the C_t (crossing threshold) values reported by other instruments, and represent the cycle at which fluorescence signal in a reaction well rises above background fluorescence signals in that well (*Figure 7*). The Cp value measured for a sample is dependent on the initial concentration of template DNA in the reaction. Lower Cp values correspond to higher initial template concentrations.

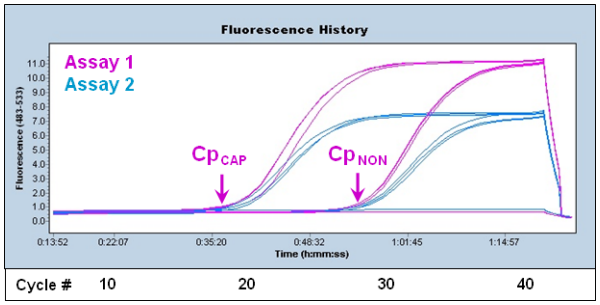


Figure 7: Example of sequence capture qPCR data for two NSC assays generated using the LightCycler® 480 Instrument II
In a successful experiment, the Cp values from qPCR of amplified captured multiplex DNA templates (CAP) will be significantly lower than Cp values from amplified sample library templates (NON) for all assays.

Analyze Data

1. Calculate the average Cp values for all replicate reactions.
2. Confirm that the negative control reactions did not produce significant fluorescent signals, which might indicate a problem with PCR contamination. Contamination could result in difficulty interpreting experimental results.
3. For each different sample and NSC assay combination, subtract the average Cp value, measured for the amplified captured multiplex DNA template, from the average Cp value measured for the corresponding amplified sample library template. This value is the ΔCp. A successfully enriched amplified captured multiplex DNA sample should generate a lower Cp value than its corresponding amplified sample library. Thus the ΔCp calculated from an NSC assay should be positive if the capture process enriched the corresponding locus.
4. Calculate the fold enrichment for a NSC control locus by raising the PCR Efficiency (*E*) for that assay to the power of the ΔCp measured for the corresponding control locus, or $E^{\Delta Cp}$.

When PCR assays operate at 100% theoretical efficiency (*i.e.* a perfect doubling of target sequences in every cycle), E = 2. The E values for the NSC assays listed in the table (below) were measured by Roche. However, because multiple parameters (PCR instrument, reagent lots, *etc.*) can affect the efficiency of a PCR assay, it is recommended to determine E values empirically in your own laboratory for each different NSC assay.

NSC qPCR Assay Name	Primer Sequences (5′ → 3′)	T_m (°C)	Product Length	qPCR Efficiency (*E*)
NSC-0237	F: CGCATTCCTCATCCCAGTATG R: AAAGGACTTGGTGCAGAGTTCAG	81.15	80 bp	1.84
NSC-0247	F: CCCACCGCCTTCGACAT R: CCTGCTTACTGTGGGCTCTTG	81.03	74 bp	1.80
NSC-0268	F: CTCGCTTAACCAGACTCATCTACTGT R: ACTTGGCTCAGCTGTATGAAGGT	78.99	75 bp	1.78
NSC-0272	F: CAGCCCCAGCTCAGGTACAG R: ATGATGCGAGTGCTGATGATG	82.23	71 bp	1.93

Example: NSC-0268 assay

(assuming E = 1.78)

- Replicate Cp values for qPCR of amplified sample library = 28.3, 28.5, 28.4
- Replicate Cp values for qPCR of amplified captured multiplex DNA = 17.5, 17.3, 17.7
  - Average $Cp_{\text{amplified sample library}} = 28.4$
  - Average $Cp_{\text{amplified captured multiplex DNA}} = 17.5$
  - $\Delta Cp = 10.9$
  - Fold enrichment $(E^{\Delta Cp}) = (1.78)^{10.9} = \mathbf{537}$

Interpreting qPCR Results – SeqCap EZ Probe Pool Captures

- Theoretical maximum average fold enrichment: Varies significantly for each SeqCap EZ experiment based on target size, from 75-fold for a 40 Mb target to 12,000-fold for a 250 kb target. Actual average fold enrichment values are typically lower than this, and different targets of the same cumulative size can enrich to different levels depending on variation in the sequence makeup of the region(s) targeted for capture and other factors. For example, if 50% of a genome were targeted for capture, an ideal result should yield no better than a two-fold average enrichment for the targeted loci.
- Correlation of fold enrichment determined by qPCR and sequencing data: Probes targeting the NSC control loci were designed using different criteria than the SeqCap EZ probes, and so their reported qPCR values should not be interpreted as a literal estimate of the expected enrichment of other targeted loci. Rather, the NSC control assays are primarily intended as a screen for potentially poor enrichment results so that unproductive sequencing may be avoided.
- Negative Control: Average Cp values for negative control (PCR-grade water) assays should be negligible, or they may indicate the presence of cross-contamination among wells or reagent contamination. If this is observed, the qPCR experiment should be repeated.
- Positive Control: Average Cp values for positive control (genomic DNA) assays should be similar (within approximately 1 Cp) to the average Cp values obtained for the qPCR of amplified sample library.
- Recommendation: Based on experiments conducted at Roche, we recommend not sequencing the captured DNA from any SeqCap EZ experiment with calculated average fold enrichment values less than tenfold because of the potential that this indicates a failed enrichment. We recommend such experiments be repeated from the beginning. Thus, the definition of a 'successful' Roche Sequence Capture experiment, as estimated by fold enrichment values for control loci, might differ substantially with different sized capture targets or different downstream applications for the captured DNA.

Appendix B. Hybridization Preparation using Vacuum Centrifugation

Appendix B describes an alternative method to the bead based hybridization preparation from *Chapter 5*, Step 5 "Prepare the Hybridization Sample". This method can be used if vacuum centrifugation is available. Which method of hybridization preparation to use, bead based or using vacuum centrifugation, will depend upon the user's preference.

This appendix requires the use of the following reagents:

- SeqCap EZ probe pool (refers to SeqCap EZ, SeqCap EZ Prime, or SeqCap EZ probes)
- HyperCap Target Enrichment Kit

1. Add 5 µL of COT Human DNA (1 mg/mL), contained in the HyperCap Target Enrichment Kit, to a new 1.5 mL tube.
2. Add 1 µg of Multiplex DNA Sample Library to the 1.5 mL tube containing 5 µL of COT Human DNA.
3. Add 5 µL of the HyperCap Universal Blocking Oligos to the Multiplex DNA Sample Library Pool plus COT Human DNA.
4. Close the lid of the tube and make a hole in the top of the tube's cap with an 18 to 20 gauge or smaller needle.

   The closed lid with a hole in the top of the tube's cap is a precaution to suppress contamination in the DNA vacuum concentrator.

5. Dry the Multiplex DNA Sample Library Pool/COT Human DNA/Universal Blocking Oligos in a DNA vacuum concentrator on high heat (+60 °C).

   Denaturation of the DNA with high heat is not problematic because the hybridization utilizes single-stranded DNA.

6. To each dried-down Multiplex DNA Sample Library Pool/COT Human DNA/Universal Blocking Oligos, add:
   - 7.5 µL of 2X Hybridization Buffer
   - 3 µL of Hybridization Component A
7. Cover the hole in the tube's cap with a sticker or small piece of laboratory tape.
8. Vortex the Multiplex DNA Sample Library Pool/COT Human DNA/Universal Blocking Oligos plus Hybridization Cocktail (2X Hybridization Buffer + Hybridization Component A) for 10 seconds.
9. Centrifuge at maximum speed for 10 seconds.
10. Transfer the Multiplex DNA Sample Library Pool/COT Human DNA/Universal Blocking Oligos/Hybridization Cocktail to the 4.5 µL aliquot of SeqCap EZ probe pool in a 0.2 mL PCR tube prepared in *Chapter 2* (the entire volume can also be transferred to one well of a 96-well PCR plate).
11. Mix thoroughly by vortexing for 10 seconds.
12. Centrifuge at maximum speed for 10 seconds.

13. Incubate in a thermocycler using the following program:

- 95 °C for 5 minutes
- +47 °C for 16 to 20 hours.

For incubation at 47 °C for 16 to 20 hours, it is important that the thermocycler's heated lid is turned on and set to maintain 10 °C above the hybridization temperature (+57 °C). The samples must remain at 47 °C until it is transferred to the capture beads in *Chapter 6*, Step 3.

Appendix C. Post-Capture Multiplexing for Sequencing

This appendix provides a recommended workflow for pooling Amplified Captured DNA Sample Libraries immediately prior to sequencing. Each DNA Sample Library contains a different DNA Adapter Index and is captured independently (*Figure 8*). Following the Post-Capture LM-PCR amplification step, the different Amplified Captured libraries are quantitated and pooled so that the pool contains equivalent amounts (by mass) of each library. To determine the appropriate number of libraries to pool, consider the capture target size, the capture specificity (*i.e.* 'on-target read rate') for the design, and your desired coverage depth.

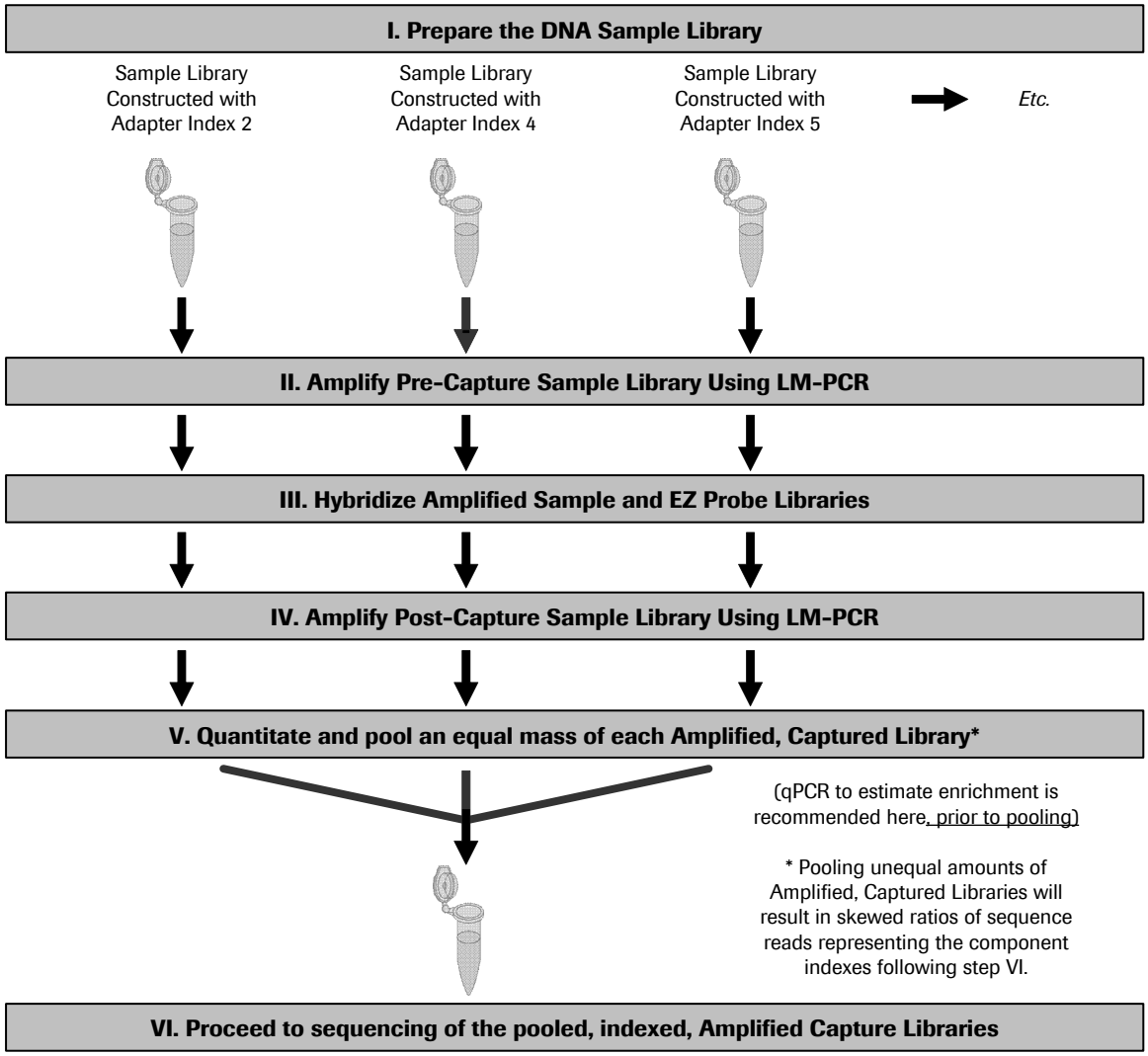
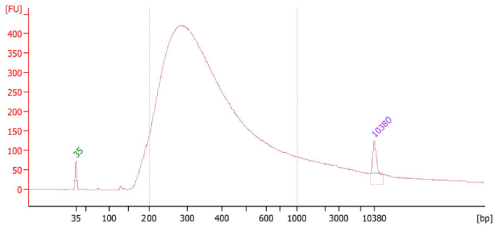
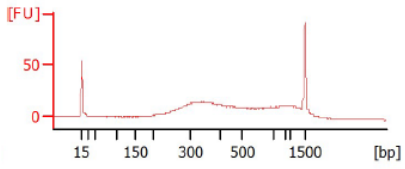
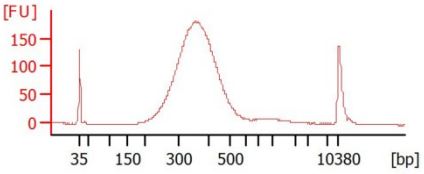


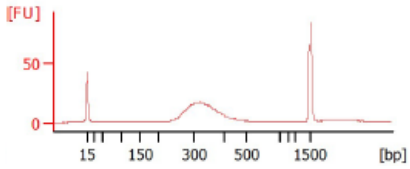
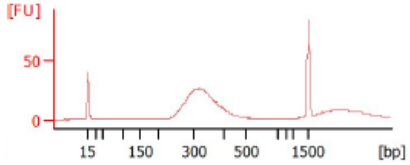
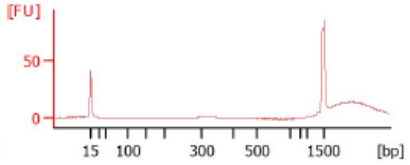
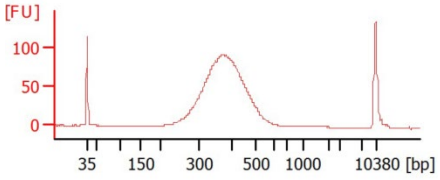
Figure 8: Post-capture multiplexing workflow

Appendix D. Troubleshooting

This appendix provides guidance for interpreting unexpected results and recommendations for implementing corrective action if problems occur. For technical questions, contact your local Roche Technical Support. Go to *sequencing.roche.com/support.html* for contact information.

Observation	Cause(s) / Recommendation(s)
Sample Library Preparation	
Less than 100 ng of input DNA is available for library preparation.	Libraries generated using <100 ng of input gDNA can produce high quality capture results; however, several adjustments summarized below, will increase the probability of success. ■ Adjust the adapter concentration to preserve the adapter: insert molar ratio in order to maintain high ligation efficiency. For more information, contact Roche Technical Support. ■ Increase the number of PCR cycles during Pre-capture LM-PCR by 1 – 3 cycles, depending on starting gDNA amount. Performance of these cycle number recommendations may vary for your particular sample. ■ Elimination of double sided size selection may improve complexity of low input sample. Elimination of double-sided size selection will result in a higher portion of large fragments, which can result in a lower on-target rate since sequencing may not extend into the targeted regions. The trade-off between metric impact and increased complexity should be considered before eliminating this step. **Note:** There is a possibility that these steps will not lead to success with lower input amounts. For the most current guidance on working with lower input amounts, contact Roche Technical Support.
DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue is available for library preparation.	DNA extracted from FFPE tissue is highly variable in quality due to chemical damage and fragmentation, and often is available only in small amounts. ■ To increase probability for success, follow recommendations for library construction using less than 100 ng of input DNA and perform enzymatic DNA fragmentation following the KAPA HyperPlus Library Preparation workflow. This can help to remove chemically-damaged termini that will interfere with adapter ligation. ■ KAPA HyperPlus library preparation is recommended since it improves sample complexity through increased adapter ligation efficiency. ■ Elimination of double sided size selection may improve complexity of low quality sample. Elimination of double-sided size selection will result in a higher portion of large fragments, which can result in a lower on-target rate since sequencing may not extend into the targeted regions. The trade-off between metric impact and increased complexity should be considered before eliminating this step. Example of FFPE sample pre-capture LM-PCR fragment distribution (analyzed using a Agilent High Sensitivity DNA chip) with elimination of double sided size selection:  **Note:** There is a possibility that these steps will not lead to success with DNA extracted from FFPE samples. For the most current guidance on working with FFPE samples, contact Roche Technical Support.

Observation	Cause(s) / Recommendation(s)
Amplified Sample Library (Pre-Capture LM-PCR Product)	
Yield is <1 µg (yield should be ≥1 µg).	Possible error occurred during library preparation or compromised reagents were used. Use a previously processed DNA sample as a positive control for library construction and or an evaluated sample library as a positive control for LM-PCR reagents.
Yield is significantly >1 µg (yield should be ≥1 µg) when amplifying Hyper Prep libraries ligated with the Dual-Index adapters.	The total number of Pre-capture LM-PCR cycles for Hyper Prep libraries containing the Dual-Index adapters can be decreased by 1 or 2 cycles of significantly more than 1 µg of DNA is obtained when amplifying with 11 total cycles.
Fragment distribution (analyzed using a Agilent DNA 1000 chip) shows that the average amplified fragment size is not within the size range of 150 to 500 bp.	Poor fragmentation occurred. Repeat library preparation.
Fragment distribution (analyzed using an Agilent DNA 1000 chip) is bimodal, with a larger set of fragments observed in addition to, or instead of, the expected set of fragments.	See the section entitled "Amplified captured multiplex DNA (Post-Capture LM-PCR Product)" in this appendix.
A_{260}/A_{280} is <1.7 (ratio should be 1.7 to 2.0).	Inefficient sample cleanup. Repeat cleanup.
The negative control yield measured by the NanoDrop spectrophotometer is non-negligible.	The measurement may be high due to the presence of oligonucleotides carried over from previous steps/LM-PCR. This carryover will be apparent as one or more sharp peaks visible less than 150 bp in size when examining the data from the Agilent Bioanalyzer DNA 1000 chip. This carryover is not a sign of contamination and will not interfere with the capture process.
The Agilent Bioanalyzer DNA 1000 chip indicates one or more visible sharp peaks that are <150 bp in size.	These peaks, which represent primers, primer-dimers or adapter-dimers will not interfere with the capture process but could lead to overestimation of the amplified library yield when interpreting the data from NanoDrop spectrophotometer. Repeat the LM-PCR cleanup.
The Agilent Bioanalyzer DNA 1000 chip indicates that the average amplified material is 150 to 500 bp in size in the negative control for sample library amplification.	This material could represent cross-contamination between amplified sample libraries. Test reagents for contamination and replace if necessary. Repeat library construction using fresh genomic DNA.
The Agilent Bioanalyzer DNA 1000 chip indicates a double peak between 200 to 1500 bp 	Possible bead carryover during the double sided size selection step. Repeat library preparation or contact technical support for guidance.
The Agilent Bioanalyzer High Sensitivity DNA chip can also be used for qualifying samples following Pre-capture LM-PCR amplification and purification.	Depending on concentration, samples may need to be diluted 1:10 prior to running on an Agilent Bioanalyzer High Sensitivity chip. An example trace is below. 
Hybridizing the Sample Library and SeqCap EZ Probe Pool	
Non-standard hybridization times.	Roche SeqCap EZ experiments can safely incubate during the hybridization step for up to 3 days (72 hours). For guidance on shorter hybridization times, contact technical support.

Observation	Cause(s) / Recommendation(s)
Amplified captured multiplex DNA (Post-Capture LM-PCR Product)	
Yield is <500 ng (yield should be ≥500 ng).	Library construction or pre-capture LM-PCR failed. Pre-capture LM-PCR yield should be ≥ 1 µg. Repeat with a DNA sample that was previously processed with success. Incorrect hybridization or wash temperatures were used. Make sure the correct hybridization and wash temperatures were used. If temperatures were not correct, repeat the experiment from hybridization. PCR reagents are compromised. Verify that the positive control worked. If the positive control did not work, repeat hybridization and re-amplify using fresh PCR reagents. **Note:** Experiments designed to capture less genomic DNA (*i.e.* a smaller cumulative target size) may be successful even though they can generate lower LM-PCR yields than experiments designed to capture larger targets. Target size and enrichment qPCR results (*Appendix A*) should be taken into consideration when evaluating low Post-Capture LM-PCR yield.
Fragment distribution (analyzed using a Agilent DNA 1000 chip) shows that the average amplified fragment size is not within the size range of 150 to 500 bp.	Poor fragmentation occurred. Repeat library preparation. Consider implementing the gel-cut size selection option.
Fragment distribution (analyzed using a Agilent DNA 1000 chip) is bimodal, with a larger set of fragments observed in addition to (Fig B), or instead of (Fig C), the expected set of fragments (Fig A): A.  B.  C. 	Primer depletion due to over-amplification of sample library relative to the amount of primers available in the reaction results in single stranded amplification products. These products can anneal to each other via adapter homology on both ends of the fragments to form heteroduplexes, and migrate as apparently much larger products on an Agilent DNA 1000 chip than their actual length in base pairs. The artifact can be resolved by increasing primer concentration or reducing cycle number in the LM-PCR reaction, however the products themselves are perfectly acceptable for use in sequence capture and sequencing, and this artifact will not affect capture performance. Care should be taken to quantify the area under both peaks if quantification will be performed using the Bioanalyzer image. *The Agilent DNA 1000 chip traces shown in Figs A, B, and C (left) show the result of amplification of the same captured gDNA sample library following post-capture LM-PCR amplification for 16, 18, or 22-cycles, respectively. The same artifact can appear in pre-capture LM-PCR amplification.*
A_{260}/A_{280} is <1.7 (ratio should be 1.7 to 2.0).	Inefficient sample cleanup. Repeat cleanup.
The Agilent Bioanalyzer High Sensitity DNA chip can also be used for qualifying samples following Post-capture LM-PCR amplification and purification.	Depending on concentration, samples may need to be diluted 1:10 prior to running on an Agilent Bioanalyzer High Sensitivity chip. An example trace is below. 

Observation	Cause(s) / Recommendation(s)
Sequencing Performance Metrics	
High Duplicate rates	Reduction in pre-capture and/or post-capture LM-PCR cycles may reduce duplicate rates. Take the following points into consideration when altering cycle numbers. ■ Enough material is present to accurately quantify after LM-PCR clean-up. ■ For the pre-capture LM-PCR, enough amplified library is produced for at least one 1ug total for hybridization. Increasing input into hybridization may improve duplicate rates. Take the following points into consideration when adjusting sample input into hybridization. ■ Enough material is produced from the pre-capture LM-PCR reaction(s) to increase sample input into hybridization. Elimination of double sided size selection may improve complexity of sample and reduce duplicate rates. Elimination of double-sided size selection will result in a higher portion of large fragments which can result in a lower on-target rate since sequencing may not extend into the targeted regions. The trade-off between metric impact and increased complexity should be considered before eliminating this step. **Note:** There is a possibility that these steps will not lead to success in reducing duplicate rates. For the most current guidance, contact Roche Technical Support.
Measurement of Enrichment Using qPCR	
Standard deviation of triplicate qPCR reactions is >0.5.	Pipetting error may have occurred. Repeat qPCR assays.
Average Cp values for negative control assays are not negligible.	Possible cross contamination across wells or reagent contamination occurred. Repeat qPCR assays.
One or more calculated NSC control locus fold enrichment values, or the average of all four, are less than the recommended value of tenfold (*Appendix A*).	Low qPCR values often correlate with low capture specificity, or a reduced 'on target' rate, for captured fragments. Repeat qPCR assays. If the result is confirmed, repeat the experiment from the beginning.

The Illumina sequencing workflow is not supported by Roche Technical Support.

Appendix E. Limited Warranty

1. Limited Warranty

A. Products: Roche Sequencing Solutions, Inc. ("Roche") warrants that its Products conform to its published specifications and are free from defects in material or workmanship. Customer's sole and exclusive remedy (and Roche's sole and exclusive liability) under this limited warranty shall be to either (a) replace the defective Products, or (b) provide Customer with a refund, as solely determined by Roche.

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